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Observations on the structural development of immature maize embryos (*Zea mays* L.) during *in vitro* culture in the presence or absence of 2,4-D

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SUMMARY

Immature embryos of two inbred lines, the Black Mexican Sweet corn (BMS) and A188, were excised from developing caryopses and cultured on nutrient media in the absence or presence of 2,4-dichlorophenoxy acetic acid (2,4-D). The morphological and cytological development was investigated by light and electron microscopy *in vitro*.

Immature BMS embryos, cultured on solidified nutrient medium without 2,4-D, exhibited premature germination and normal plant development, provided the excised embryos were older than 9 days after pollination (DAP), the scutellum was in contact with the nutrient medium and the endosperm was removed completely. Embryos failed to germinate and exhibited deformations in roots and shoots when excised before 9 DAP and when the axis side was placed onto the medium or when the embryos were still surrounded by endosperm.

The culture of immature BMS and A188 embryos on solidified media in the presence of 2 mg 2,4-D/l induced reactions in both embryo axis and scutellum such as cell degeneration, differentiation of chlorenchyma, collenchyma and vascular tissue and cell multiplication. Callus formation and the development of somatic embryos or adventitious shoots were observed at the basal end of the scutellum, provided the axis side of the embryo was in contact with the medium. The regeneration capacity of the inbred line A188 was highest. Nearly all embryos regenerated up to 15 adventitious shoots or somatic embryos which developed from cell clusters with a presumable single cell origin.

Suspension cultures were established from both inbred lines. Cell divisions were, however, exclusively found in root tip-like aggregates. Somatic embryogenesis and organogenesis were not observed in liquid media.

Abbreviations: BMS = Black Mexican Sweet corn, 2,4-D = 2,4-dichlorophenoxy acetic acid, DAP = Days after pollination, DIC = Days in culture, GP-2 = Medium according to Green & Phillips (1975), supplemented with 2 mg 2,4-D/l, LM = Light microscopy, SEM = Scanning electron microscopy, TEM = Transmission electron microscopy.

Key-words: callus, 2,4-D, somatic embryogenesis, structure, suspension culture, Zea.

INTRODUCTION

In vitro techniques are often applied when deficiencies in sexual reproduction lead to low seed setting or embryo abortion. The culture of immature embryos prevents embryo abortion when endosperm development retards or fails (Sheridan *et al.* 1978). Moreover, embryo culture provides a tool for the initiation of embryogenic callus (Street 1977, Sheridan 1982, Evans *et al.* 1983, Vasil 1984).

With maize, the culture, germination and subsequent development of immature embryos can be established in the absence of 2,4-D, as shown by Mock & Dahmen (1973) and Sheridan *et al.* (1978). In the present report the germination ability of variously aged immature embryos is related to the developmental stage of the embryo at the onset of culture as described for the *in vivo* embryogenesis (see Van Lammeren 1986b). The tissues surrounding the embryo were removed as much as possible to reduce uncontrolled influences. In order to select appropriate environmental conditions several media were tested.

The culture of immature embryos in the presence of 2,4-D resulted in callus formation and somatic embryogenesis on solidified medium (see, e.g. Green & Phillips 1975, Vasil *et al.* 1983, 1984, Duncan *et al.* 1985) and to the establishment of a cell suspension in liquid media (Vasil 1982, Green *et al.* 1983, Imbrie-Milligan & Hodges 1986). The structural aspects of callus formation and embryoid differentiation were studied by, amongst others, Springer *et al.* (1979), Vasil (1982) and Vasil *et al.* (1985). In this paper additional observations with scanning electron microscopy, transmission electron microscopy and light microscopy on scutellum differentiation and embryoid development are presented and compared with the cytological and morphological data of *in vivo* embryogenesis (Schel *et al.* 1984, Van Lammeren 1986 a,b, Van Lammeren & Schel 1983). Additionally, cell suspensions were established in order to have a single cell system to investigate callus formation, regeneration and somatic embryogenesis.

Two inbred lines of *Zea mays* L. were used. Line A188, kindly provided by C.E. Green (University of Minnesota, St Paul, USA), was selected because of its potency to form differentiating cultures and to regenerate plants *in vitro* (Green & Phillips 1975).The BMS, kindly provided by R. J. Lambert (University of Illinois, Illinois, USA), was chosen because of its potency to produce callus and to initiate good cell suspensions (Sheridan 1975, 1977). Premature germination was investigated with inbred line BMS, callus growth and plant regeneration mainly with A188, whereas the suspension culture was performed with both lines.

MATERIALS AND METHODS

Plants were grown under greenhouse conditions, with 16 h light at 23°C and 8 h darkness at 18°C. Cobs were masked with small bags before the emergence of the silks which were hand pollinated.

Ovaries of BMS and A188 were dissected at defined intervals from 1 day after pollination (DAP) onwards, sterilized according to Dahmen & Mock (1972) and rinsed in sterile water. Embryo sacs surrounded by nucellus tissue and blocks of endosperm containing

the embryos were dissected when the embryos were too small to manipulate, i.e. from 1 up to 6 DAP. Older embryos were either dissected from the endosperm or dissected with some attached endosperm and placed on the culture medium solidified with 0·7% agar noble (Difco). Various nutrient media were tested: the B5 medium of Gamborg *et al.* (1968), the media of Graebe & Novelli (1966), of Green & Phillips (1975) and of Monnier (1976). Germlings, developed *in vitro*, were allowed to mature in a mixture of *Sphagnum* and sand (1:1) moistened with a 0·1% solution of chemical manure. Embryos were prepared for electron microscopy (SEM, TEM) and light microscopy (LM) essentially as reported previously (Van Lammeren 1986b). Embryos that were prepared for SEM were post-fixed in 1% OsO_4 in 0·1 M Na-cacodylate buffer, pH 7·0.

Suspension cultures were initiated from dissected BMS and A188 embryos (13-15 DAP) in a medium according to Green & Phillips (1975) supplemented with various concentrations of 2,4-D ranging from 0·5 to 2·0 mg/l. Suspensions were cultured in spinner flasks or on an orbital shaker (140 rev/min at 27°C and 16 h light, 2200 lux). Viability tests on suspension cells were performed with fluorescein diacetate (FDA, see Heslop-Harrison & Heslop-Harrison 1970), with lissamine green and by observing cytoplasmic streaming. Lissamine green is a selective stain for the cytoplasm of degenerating and degenerated cells because it only permeates disrupted cell membranes. A 0·1% solution of lissamine green in culture medium was prepared and stored at −20°C. It was diluted 1:50 with culture medium just before staining and one drop was added to an equal volume of cell suspension on a slide; observations were made after 5 min.

RESULTS

Premature germination in the absence of 2,4-D

Depending on the age of the excised BMS embryo, the presence of endosperm and the composition of the culture medium, various growth responses were observed on media without 2,4-D. Embryo sacs in sagittal sections of ovaries and surrounded by nucellus cells were set in culture at 1 DAP and developed callus at the site of the zygote. Callus growth ceased after 3 days and no root or shoot meristems were formed. If older embryos were surrounded by some endosperm they exhibited a different type of development compared with the embryos which were dissected without endosperm (Table 1). Root formation was observed in the cultured pro-embryos excised at 3 DAP. These pro-embryos had no root meristems initially (Van Lammeren 1986b). Embryos which were excised between 7 and 16 DAP and which were still embedded in endosperm developed morphologically normal roots but only small defective shoots.

The culture of embryos that were free from endosperm resulted in less deformations of the root and the shoot especially on the medium of Graebe & Novelli which was therefore selected as the nutrient medium. Embryos excised at 6–7 DAP showed normal root formation but limited shoot development. When embryos of 9 DAP and older were excised from the endosperm and cultured on the medium of Graebe & Novelli without 2,4-D, the culture resulted in premature germination provided that the abaxial side of the scutellum was in contact with the nutrient medium. Compared with the *in vivo* development, the growth of the scutellum retarded, whereas the development and elongation of roots and shoots accelerated. These plantlets were grown to maturity and produced normal inflorescences.

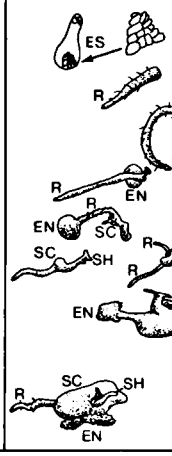
EMBRYO AGE (DAP)	MEDIUM	EMBRYO DEVELOPMENT								
		EMBEDDED IN ENDOSPERM					EXCISED FROM ENDOSPERM			
		R/SH/DEF			IES	DRAWINGS	R/SH/DEF		IES	DRAWINGS
1	G&N	-	-	+	0.1					
3	MONN.	+	-	-	0.2					
5-6	G&N	+	*	-	0.4		+	*	-	0.4
7-8	"	+	-	+	0.6		+	-	-	0.8
8-9	G-B5	+	-	+	0.8		*	*	+	1.4
9	G&N	+	+	+	0.8		+	+	-	1.5
11	"	+	+	*	1		+	+	+	1
13	"	-	-	+			+	+	-	0.8
15-16	"	+	+	+	2		+	+	-	1.5

Table 1. *In vitro* development of immature maize embryos (strain BMS) which were excised at various days after pollination (DAP). The embryos were either still embedded in endosperm (first series of drawings) or excised from the endosperm (second series of drawings) and cultured without 2,4-D on the nutrient media of Graebe & Novelli (G&N, 1966), Gamborg *et al.* (G-B5, 1968) or Monnier (MONN. 1976). The last two media were only used incidentally because the best results were obtained with the G & N medium. The drawings show that a minimal embryo size or minimal embryo age and the absence of endosperm are the prerequisites for premature germination. 1) An embryo of 9 DAP which was excised and transplanted upon the endosperm of another caryopsis developed with less deformation. 2) These embryos were grown to maturity and developed fruit-bearing inflorescences. Abbreviations: (DAP) age of embryo in days after pollination at the onset of culture, (DEF) deformation, (EN) endosperm, (ES) embryo sac, (IES) initial embryo size, (R) root, (SC) scutellum, (SH) shoot, (+) distinct development, (★) limited development, (−) no development.

Callus formation and plant regeneration from embryonic tissues

Callus formation. The morphological development of callus on A188 embryos, excised at 14 DAP and cultured over a period of 45–160 h on Green & Phillips medium supplemented with 2 mg 2,4-D/l (GP-2), is shown in Fig. 1. Various growth responses were discerned; either the whole scutellum enlarged (Fig. 1a) or a local swelling was formed at the base of the scutellum (Fig. 1b). Incidentally both phenomena were observed (Fig. 1c). Sometimes only the embryo axis was swollen (Fig. 1d). All these phenomena, however, could appear simultaneously as well (Fig. 1e).

Callus was mainly observed in the basal region at the abaxial side and the lateral sides of the scutellum. Calli appeared as wart-like structures, as globular aggregates with epidermal-like smooth surfaces, or as loose, friable arrangements. Callus on the scutellum grew fast and often had a friable appearance. Callus formation on roots was observed as soon as the roots came into contact with the nutrient medium. Cell proliferation occurred in the epidermis and cortex of the seminal roots. The aggregates of callus branched frequently, due to the deficient formation of lateral roots. Adventitious roots were generated from the embryo shoot and from meristematic regions in the scutellum. They had a normal appearance and calli were not formed as long as the roots were not in contact with the nutrient medium.

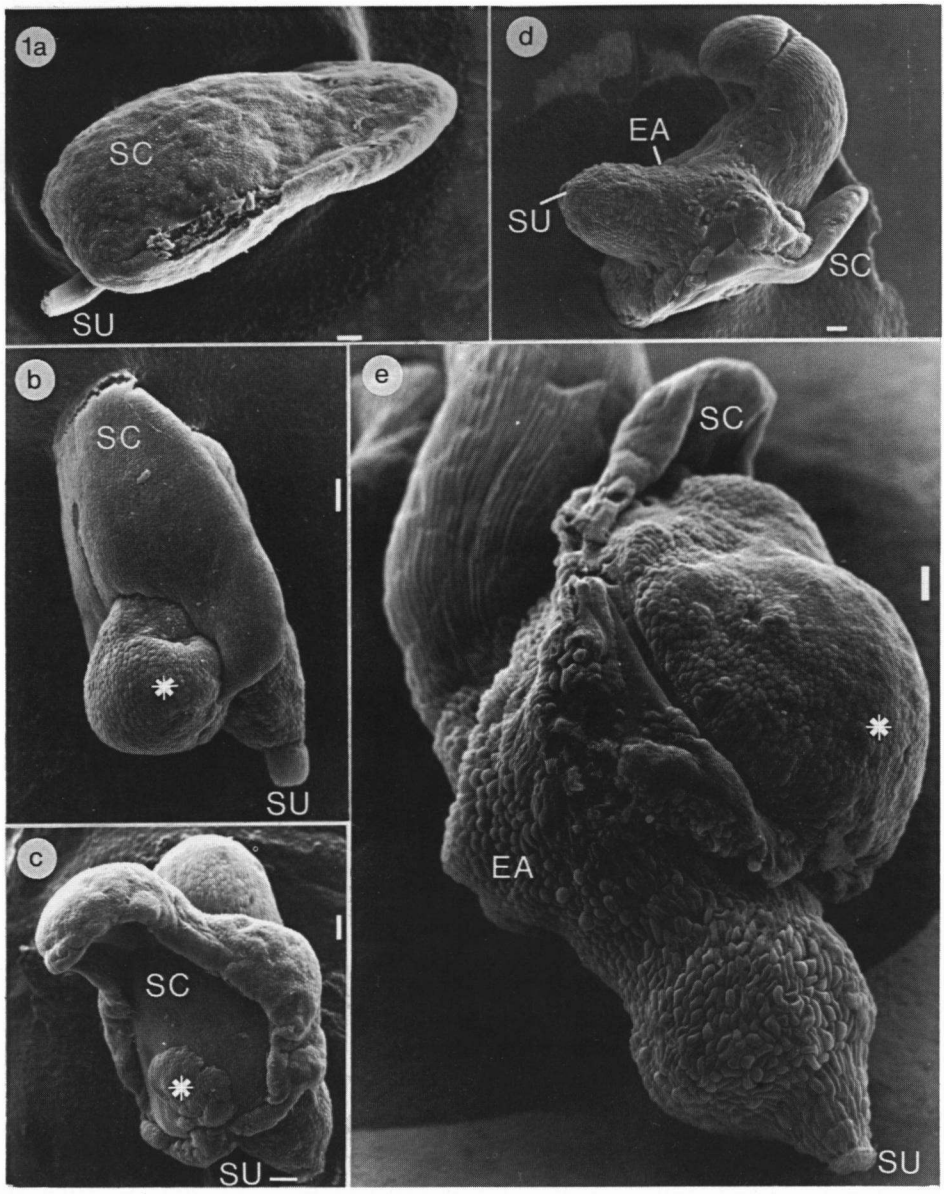


Fig. 1. Micrographs showing morphological development of callus on embryos of maize (strain A188), cultured over a period of 45–160 h on GP-2 medium. Culture period for (a) 45 h, (b–c) 68 h, (d) 90 h and (e) 160 h. Note the total enlargement of the scutellum and the embryo axis (EA) and the local swelling (*) at the base of the scutellum. The bars represent 100 μm. (SC) scutellum, (SH) shoot, (SU) suspensor.

The onset of cell proliferation in the root meristem and in the scutellum was observed within 24 h of culture. The cytological development is shown in Fig. 2. Initially, the cells of the root meristem were highly vacuolated and showed nuclei with much heterochromatin (Fig. 2b), whereas after 24 h of culture vacuoles decreased in size and nuclei became euchromatic (Fig. 2c). After 45 h in culture most vacuoles had disappeared and cells

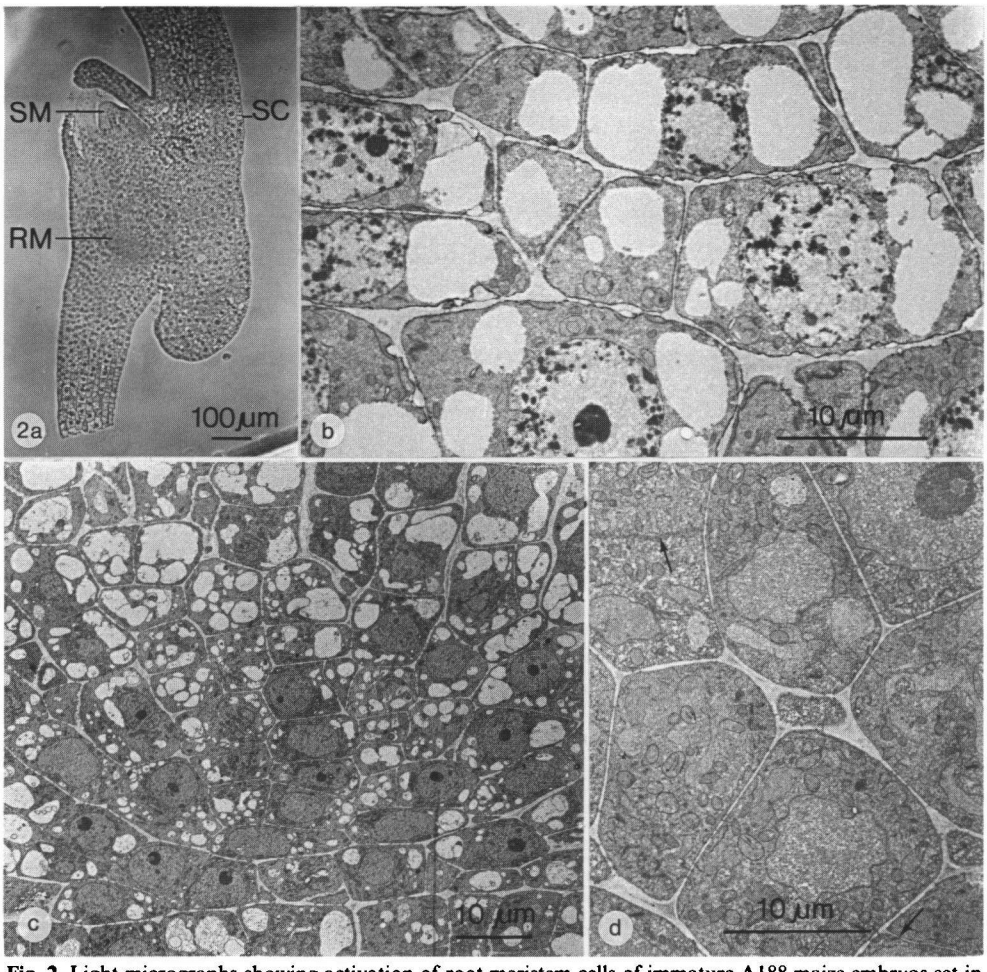


Fig. 2. Light micrographs showing activation of root meristem cells of immature A188 maize embryos set in culture on GP-2 medium with the axis side in contact with the medium. (a) A sagittal section of an embryo grown *in vivo* for 12 days. (b) The root meristem of such an embryo before culture. (c) A root meristem at 24 h and (d) the root meristem after 45 h in culture. Note the decrease in vacuolar size, the changes in the nucleoplasm and the increase in cell divisions (arrows). (SC) scutellum, (SM) shoot meristem, (RM) root meristem.

divided frequently (Fig. 2d). The swelling of the scutellum was caused by the growth of mesophyll cells and by the enlargement of intercellular spaces as visualized in Fig. 3a, b and c. In addition, meristematic regions were formed in the inner part of the scutellum and new strands of vascular tissues were observed (Fig. 3d, arrow). The subepidermal cells of the scutellum often developed into chlorenchyma (Fig. 3b and c) or collenchyma (Fig. 3d, arrow-heads) and in some regions they showed meristematic activity. In the meristematic regions, clusters of relatively small cells were observed (Fig. 3e). These cells had few vacuoles and many well-stained organelles and were characterized by proliferating ER and large euchromatic nuclei with conspicuous nucleoli. The clusters constituted embryoids or shoot primordia. Because of the shape of the cell clusters, such as shown in Fig. 3e, and the orientation of the cell walls in the cluster in particular, such an embryoid presumably originates from a single cell. The bordering cells of callus tissue showed less pronounced organelles and less active nuclei. On the other hand, subepidermal cells at the

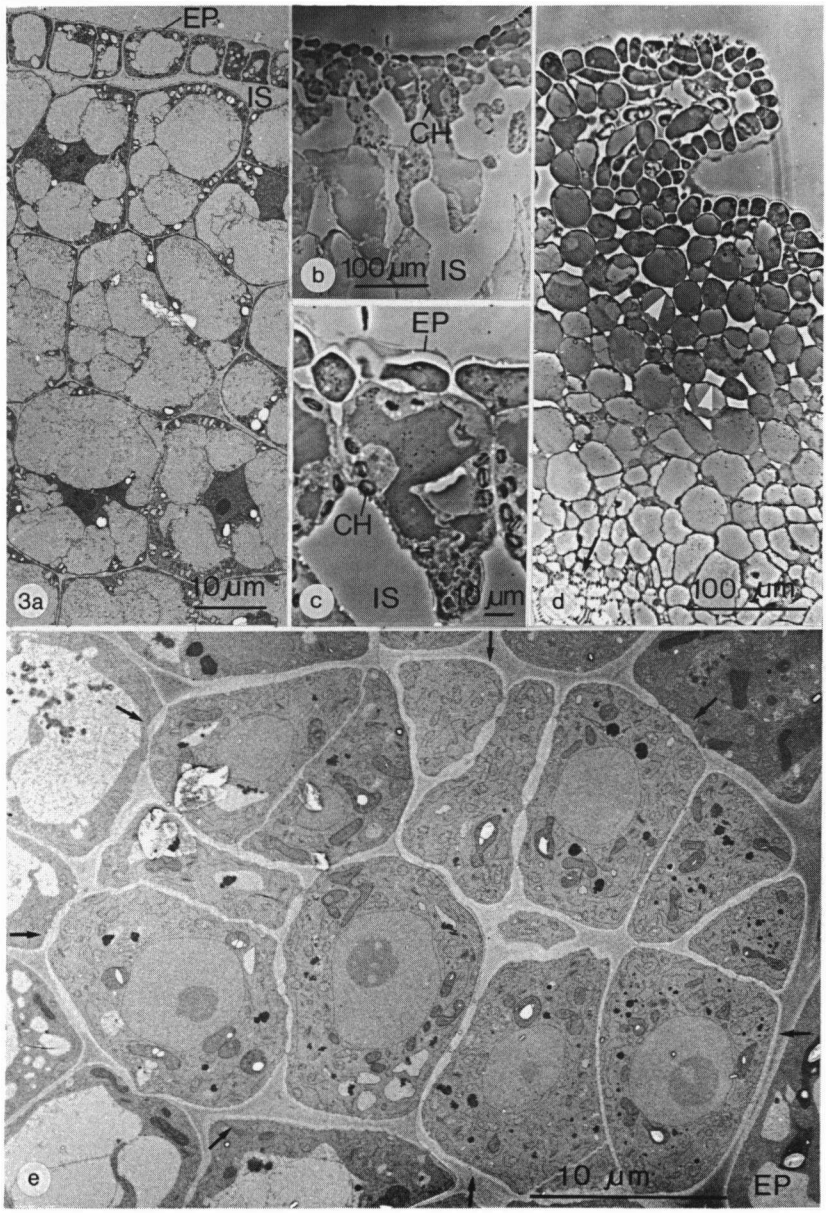


Fig. 3. Micrographs showing cytological differentiation in the maize scutellum during *in vitro* culture on GP-2 medium (strain A188). Intercellular spaces (a,b), chlorenchyma (b,c), collenchyma (d, arrow-heads), vascular tissues (d, arrow) and meristematic, callus and shoot or embryoid forming zones (e) resulted within 11 days in culture (DIC). The arrows in (e) point towards the original outline of the presumed single cell. (a) 1 DIC, (b–d) 11 DIC, (e) 13 DIC. (CH) chloroplast, (EP) epidermis, (IS) inter cellular space.

abaxial scutellum side could also degenerate within 24 h of culture and at 45 h in culture the larger part of the scutellum could then have collapsed (cf. Fig. 1d) because of cell degeneration.

In order to compare the callus-generating ability of the various scutellum regions, some embryos of 21 DAP were cut in several transversal parts and cultured on GP-2 medium.

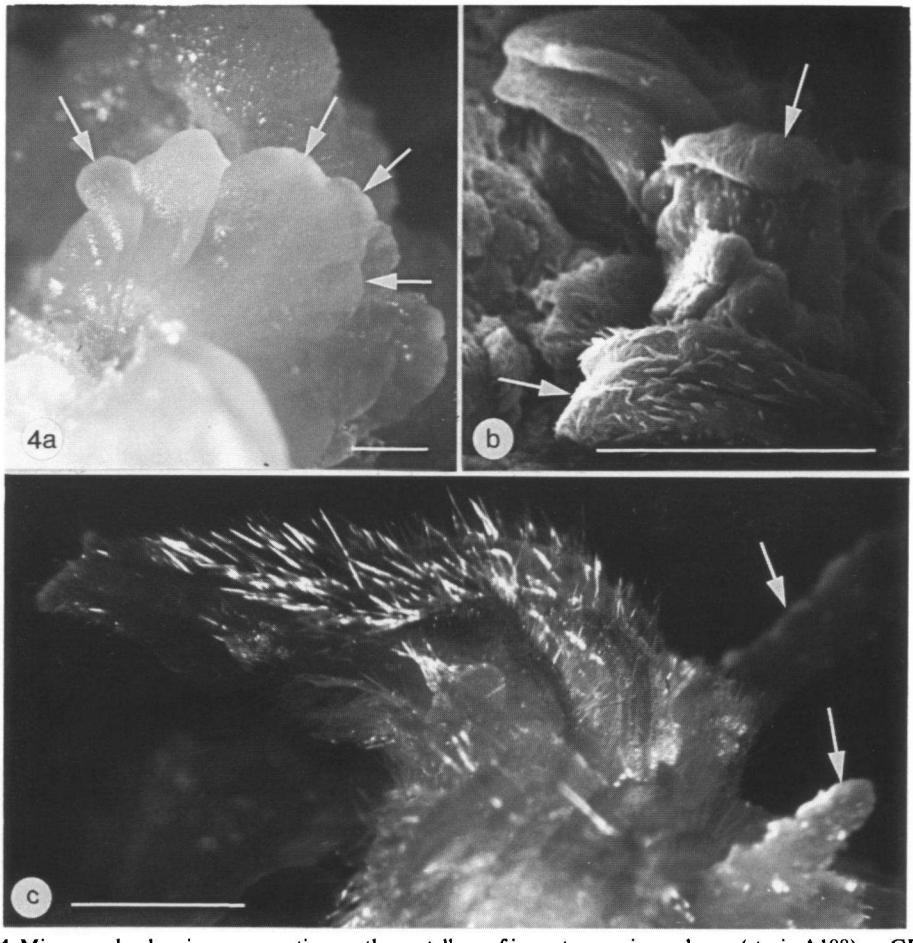


Fig. 4. Micrographs showing regeneration on the scutellum of immature maize embryos (strain A188) on GP-2 medium. (a) Formation of somatic embryos or adventitious shoots (arrows) at 25 DIC. (b–c) Differentiating leaflets of adventitious shoots at 38 DIC. The adventitious roots (c, arrows) do not originate from the adventitious shoots. The bars represent 1 mm.

Except for the one containing the apex of the scutellum, all sections had formed callus after 4 days in culture.

Plant regeneration. After a culture period of 3 weeks on solidified GP-2 medium, somatic embryos or adventitious shoots (Fig. 4a, arrows) and leafy structures (Fig. 4b and c) developed in the basal region at the abaxial side of the scutellum of A188 embryos. Their place of origin was not fixed but the epidermis at such a site was undisrupted initially. At the beginning of differentiation somatic embryos or adventitious shoots often had a scutellum-shaped appearance with a shoot meristem-like structure at the surface. The leafy structures bore many epidermal hairs (Fig. 4c). Adventitious roots developed (Fig. 4c, arrows) but were never seen to originate from the adventitious shoots under the applied conditions. Embryos of strain BMS formed leafy structures after about 6 weeks. Compared with BMS, strain A188 also formed more adventitious roots and shoots. Nearly all the cultured embryos of strain A188 produced up to 15 adventitious organs

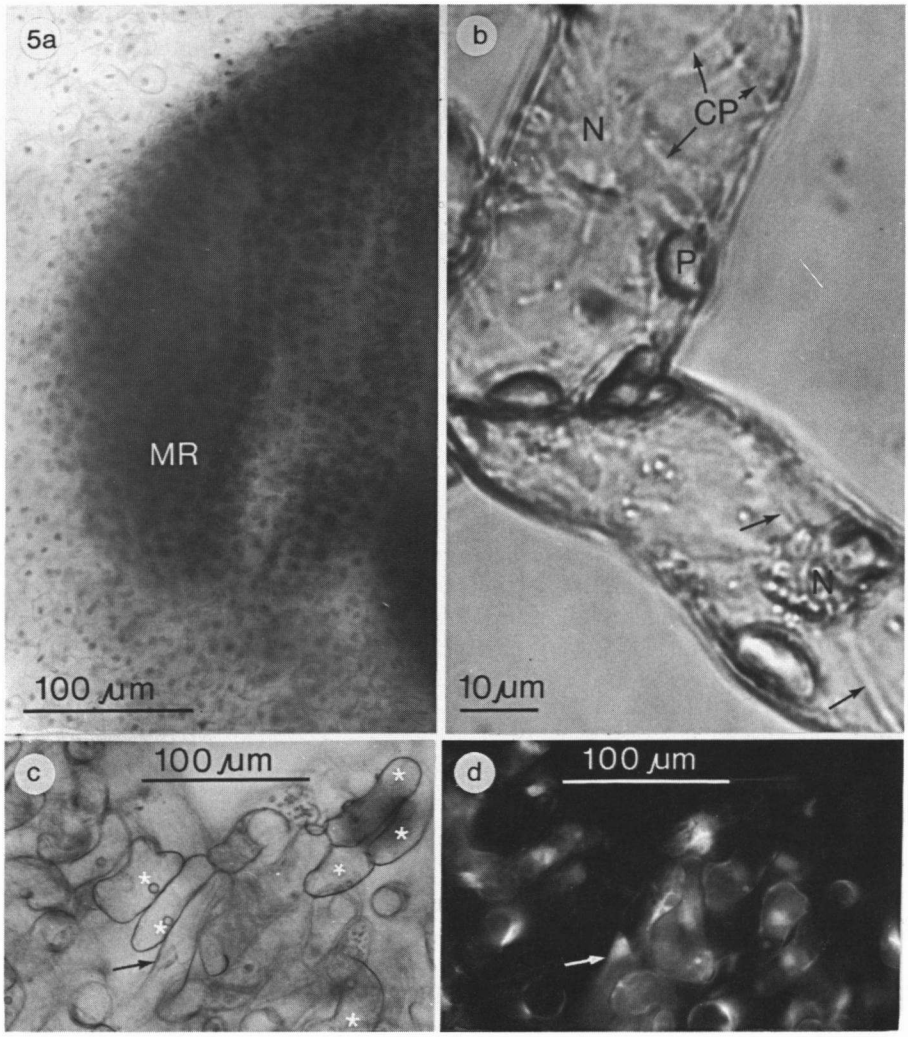


Fig. 5. Micrographs showing maize cells in liquid medium. Culture of immature embryos (strain A188) in liquid GP-2 medium resulted in the development of a cell suspension containing cell aggregates (a) with meristematic regions (MR). Cell production was dependent on the presence of cell aggregates in both strains. (b–d) Determination of viability in cell suspensions derived from immature maize embryos of strain A188. The viability was either determined by observing cytoplasmic streaming and the integrity of the cytoplasm (b) or by staining with lissamine green (c) and fluorescein diacetate (d). In the phase contrast view (c) some disintegrated and lissamine green accumulating cells are marked (*). The dark-field micrograph (d) of the same cells shows exclusively the 'living', FDA-positive cells. The black and white arrows in pictures (c) and (d) point towards the same nucleus. (CP) strand of cytoplasm, (N) nucleus, (P) plastid.

each in a culture period of 3 months, whereas only up to 20% of the BMS embryos bore adventitious shoots. It was observed that wart-like structures often developed into adventitious roots.

Culture in liquid media

Embryos of BMS and A188 were dissected at 16 DAP and cultured in liquid GP medium supplemented with various concentrations of 2,4-D. When cultured without 2,4-D, the

embryos formed roots and shoots. After the addition of 0·75 mg 2,4-D/1, the scutellum enlarged, the coleoptile partly developed and root formation was inhibited. Some small clusters of cells were detected in the suspension fluid. When cultured at 2 mg 2,4-D/1, there was a slight growth of the embryo axis, the scutellum enlarged and callus was formed within 5 days. At 8 days in culture, individual cells and clusters of cells from both inbred lines were detected in the culture medium. With strain BMS, over 90% of the suspension cells exhibited cytoplasmic streaming after 8 days in culture. With strain A188, however, hardly any cell showed this tendency although many strands of cytoplasm radiated from the nucleus towards the periphery of the cell (Fig. 5b, arrows). During a culture period of 4 weeks a dense suspension developed. The cytoplasmic streaming-based viability decreased to about 40% with BMS and after 7 weeks of culture without dilution, cytoplasmic streaming was only observed in about 10% of the cells. Based on the lissamine green-FDA tests, however, a viability of about 30% was still detected in those cells. From the beginning of culture, the A188 cells showed cytoplasmic streaming in less than 5% but lissamine green-FDA tests, such as shown in Fig. 5c and d, gave up to 50% viability after 8 weeks. Subculturing of that suspension in GP-1 medium and a subsequent incubation of 8 days resulted in a remarkable increase in cell number and viability which exceeded 90%. Cell divisions were never observed in the suspended single cells. They were exclusively and frequently found in cell aggregates of 1·5–3·0 mm size which were not in the inoculum initially. Figure 5a shows such a cell aggregate which has a root-like appearance and might have originated from smaller cell clumps which segregate from the embryo. The increase in cell number was only observed when the cell aggregates were present.

DISCUSSION

Embryo development in vitro

Immature embryos were excised and cultured *in vitro* without 2,4-D to investigate their ability to develop in the absence of surrounding endosperm and somatic tissues. The excision and culture of a fertilized embryo sac (1 DAP, see Van Lammeren 1986a) permitted investigation of *in vitro* embryogenesis *ab ovo*. The procedure applied offered the opportunity to observe morphological differentiation continuously and to induce germination under defined conditions. Several authors have cultured embryos and achieved complete plant development from 11 DAP onwards (e.g. Mock & Dahmen 1973, Sheridan *et al.* 1978). In the present experiments, using the medium as described by Graebe & Novelli (1966), the development of plantlets from immature excised embryos was achieved from 9 DAP onwards. Compared with the *in vivo* embryogenesis (see Van Lammeren 1986b) a decreased scutellum development is obvious and an activation of meristematic activity and cell elongation resulted in early root and shoot formation. Germination only occurred when the scutellum was in contact with the nutrient medium which emphasizes the importance of the direction of the nutrient flow. Compared with the *in vivo* development, where most nutrients are taken up through the abaxial epidermis of the scutellum (Van Lammeren 1986b), a similar pathway for nutrients is evident. Embryos, set in culture at 9 DAP while surrounded by some endosperm, showed limited shoot and scutellum formation. This finding stresses the function of the endosperm to suppress germination.

Embryos younger than 9 DAP exhibited growth as well, but deficiencies occurred. When pistils were pollinated *in vivo* then excised at 1 DAP and cultured *in vitro*, the embryo and

endosperm developed (Schel & Kieft 1986). When pistils, however, were pollinated *in vivo* and sagittal sections of the ovaries containing the fertilized embryo sacs were set in culture at 1 DAP, the culture resulted in deficient embryo development indicating that this type of injury strongly influences embryo development. *In vivo* and in the experiments of Schel & Kieft (1986), the cells of the placento–chalazal region and the synergids function in the transport of metabolites and inorganic compounds towards the nucellus and the embryo, respectively (see also Van Lammeren 1986a). The endosperm cells of the placento–chalazal region and those near the embryo suspensor do function similarly (see Schel *et al.* 1984). When, however, an ovary is sectioned sagittally and the section side is placed onto the nutrient medium, the nutrients are taken up through the section surface rather than through the spikelet axis. Consequently, the composition of the nutrient flow is changed and this appears to influence embryogenesis.

Formation of cell suspensions and callus and regeneration of somatic embryos

Embryos of BMS and A188 were cultured in liquid media to form cell suspensions which might give rise to somatic embryos from single cells. The single cell stage gives the particular opportunity to study the development of structural polarity such as induced in the egg cell-zygote stages *in vivo* (Van Lammeren 1986a). A cell suspension was established but organogenesis was not observed. Cell divisions were exclusively observed in meristematic, root-like cell clusters. It is not likely that these cell aggregates originated from single cells in the medium (cf. Imbrie-Milligan & Hodges 1986) because cell divisions were never observed in single cells. It is suggested that they segregate from the proliferative embryos in the medium.

For testing the viability of suspension cells two procedures were used. It appears that cytoplasmic streaming is not a reliable characteristic compared with the FDA-lissamine green test. The FDA test, however, indicates the presence of esterase activity rather than screening the viability and should indeed be applied in combination with lissamine green.

The regeneration of somatic embryos and plantlets from callus cultures of *Zea mays* L. on solid nutrient media has been achieved from various tissues such as stem tissue from seedlings (Harms *et al.* 1976) and from excised immature embryos (see, amongst others, Green & Phillips 1975, Lu *et al.* 1983, Novak & Dolezelova 1983, Vasil *et al.* 1984, 1985, Duncan *et al.* 1985, Kamo *et al.* 1985). In the present experiments the culture of immature embryos on solidified nutrient media resulted in callus formation at various sites of the embryo. On the scutellum it was mainly restricted to the basal end which may be related to the high levels of plant growth regulators presumed to be present at this site (Vasil & Vasil 1982). The induced bipolarity existing within the scutellum, which is regarded as a leaf (Van Lammeren 1986b), was also assigned by the transversal sectioning of the scutellum; most sections exhibited callus formation. Anatomically, callus formation was found in the epidermal and sub-epidermal zones (see also Springer *et al.* 1979, Vasil *et al.* 1985).

Callus formation is only one growth response to *in vitro* culture. The swelling of the scutellum was caused by cell enlargement, the formation of intercellular spaces and, to a lesser extent, by the formation of chlorenchyma, collenchyma and vascular tissues. Only the compact scutellar callus which was formed at the basal end showed regenerative capacity. Vasil *et al.* (1983) also reported embryoid formation in the nodal region of the scutellum, but there the scutellar side of the embryo was in contact with the nutrient medium. The areas with cells dividing frequently also bore the bipolar, pro-embryo-like structures. These structures showed a unicellular origin as reported for *Pennisetum* embryoids (Vasil & Vasil 1982, Botti & Vasil 1984). In our experiments the development of

leafy structures and adventitious shoots was observed (cf. Green & Phillips 1975, Springer *et al.* 1979) rather than the formation of somatic embryos as reported by, amongst others, Lu *et al.* (1983), Novak & Dolezelova (1983), Vasil *et al.* (1984, 1985) and Fransz & Schel (1987).

In conclusion, it can be stated that excision and premature germination in the absence of 2,4-D is a useful tool to investigate morphogenesis of the embryo continuously and under experimental conditions and to obtain flowering plants from immature embryos. For maize the differentiation of meristems appears to be a prerequisit. Embryo culture in the presence of 2,4-D results in complex morphogenesis, histogenesis and cell differentiation. Histological and sub-microscopical research give evidence for the single cell origin of cell clusters which will form somatic embryos or adventitious shoots.

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